A common variant in *BRCA2* is associated with both breast cancer risk and prenatal viability

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Inherited mutations in the gene *BRCA2* predispose carriers to early onset breast cancer, but such mutations account for fewer than 2% of all cases in East Anglia. It is likely that low penetrance alleles explain the greater part of inherited susceptibility to breast cancer; polymorphic variants in strongly predisposing genes, such as *BRCA2*, are candidates for this role. BRCA2 is thought to be involved in DNA double strand break-repair[1,2]. Few mice in which *Brca2* is truncated survive to birth; of those that do, most are male, smaller than their normal littermates and have high cancer incidence[3,4]. Here we show that a common human polymorphism (N372H) in exon 10 of *BRCA2* confers an increased risk of breast cancer: the HH homozygotes have a 1.31-fold (95% CI, 1.07–1.61) greater risk than the NN group. Moreover, in normal female controls of all ages there is a significant deficiency of homozygotes compared with that expected from Hardy-Weinberg equilibrium, whereas in males there is an excess of homozygotes: the HH group has an estimated fitness of 0.82 in females and 1.38 in males. Therefore, this variant of BRCA2 appears also to affect fetal survival in a sex-dependent manner.

In an initial study to investigate whether common *BRCA2* variants alter the risk of breast cancer in the general population, we carried out an association study on six *BRCA2* polymorphisms identified through the BIC database (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/BIC; Table 1). The genotype distributions of both the exon 10 N372H and the 5′ UTR a-26g polymorphisms approached significant differences between cases and controls in our initial hypothesis-generating study. We thus restricted studies in further case-control series to confirmation of these observations, however, only the N372H findings were confirmed. N372H is the sole *BRCA2* variant resulting in an amino

Table 1 • Breast cancer risks associated with polymorphisms in *BRCA2*

Breast cancer risks due to *BRCA2* polymorphisms in case-control series 1

Polymorphism	Rare allele frequency (controls)	OR (95% CI) (common hom. reference category)	OR (95% CI) (het. versus common hom.)	OR (95% CI) (rare hom. versus common hom.)
a-26g (5′ UTR)	0.281	1.00 (0.80–1.25)[a]	0.64 (0.49–0.84)[a] *(0.45–0.91)*	0.92 (0.49–1.71)[a] *(0.47–1.77)*
N289H	0.026		1.24 (0.58–2.66)	◆
N372H	0.265	1.00 (0.78–1.28)[a]	1.02 (0.78–1.34)[a] *(0.71–1.48)*	1.74 (0.86–3.52)[a] *(0.82–3.67)*
T1915M	0.053		0.36 (0.11–1.19)[b]	◆
R2034C	0.007		0.70 (0.21–2.31)	◆
K3326X	0.007		1.71 (0.42–6.92)	◆

Breast cancer risks associated with N372H in all case-control series

Series	Rare allele frequency (controls)	OR (95% CI)[c] (NN versus NN reference category)	OR (95% CI) (NH versus NN)	OR (95% CI) (HH. versus NN)
1 British[d]	0.265	1.00 (0.78–1.28)	1.02 (0.78–1.34) *(0.71–1.48)*	1.74 (0.86–3.52) *(0.82–3.67)*
2 British	0.269	1.00 (0.90–1.11)	0.99 (0.88–1.11) *(0.85–1.15)*	1.38 (1.05–1.82) *(1.03–1.86)*
3 British	0.250	1.00 (0.80–1.24)	1.01 (0.78–1.29) *(0.72–1.40)*	1.79 (0.88–3.62) *(0.85–3.74)*
4 German	0.285	1.00 (0.87–1.15)	1.02 (0.88–1.20) *(0.83–1.27)*	1.09 (0.74–1.59) *(0.73–1.63)*
5 Finnish	0.221	1.00 (0.85–1.18)	1.04 (0.83–1.30) *(0.79–1.38)*	1.07 (0.61–1.87) *(0.60–1.92)*
Overall		1.00 (0.94–1.07)	1.00 (0.93–1.08) *(0.91–1.12)*	1.31 (1.09–1.58) *P*=0.026[e] *(1.07–1.61)*

[a]Floating CIs (for comparison, standard CIs are shown in italics; ref. 15). [b]Carrier versus non-carrier. w, no rare Hom. observed; genotyping methods available on request. [c]Floating CIs are given[15] (for comparison, standard CIs are shown in italics). [d]As above. [e]*P* value for likelihood ratio test comparing H_0 [OR(NH) = OR(HH)=1] versus H_1[OR(NH) and OR(HH) estimated and assumed to be the same in each population].

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Table 2 • N372H genotype distributions, deviation from HWE and relative fitnesses of the genotypes in adult female control sets and newborns

Series		NN	NH	HH	Relative fitness (NN versus NH)	Relative fitness (HH versus NH)	Deviation from HWE
1 British	observed	138	115	13	0.87	0.63	$P = 0.074$
	expected	143.69	103.63	18.59			
2 British	observed	631	493	77	0.94	0.85	$P = 0.137$
	expected	641.14	472.72	87.14			
3 British	observed	124	94	10	0.88	0.64	$P = 0.133$
	expected	128.25	85.50	14.25			
4 German	observed	433	373	60	0.92	0.81	$P = 0.090$
	expected	443.16	352.67	70.16			
5 Finnish	observed	277	152	24	1.03	1.11	$P = 0.599$
	expected	275.08	155.85	22.08			
newborn females	observed	590	485	75	0.93	0.83	$P = 0.062$
	expected	602.66	459.68	87.66			
all females	(composite test)				–	0.82	$P = 0.001$*
newborn males	observed	644	435	116	1.15	1.38	$P = 0.001$
	expected	621.07	480.85	93.07			

*Estimated by likelihood-ratio test: H_0 (all populations in HWE [estimate 6 genotype frequencies]) versus H_1 (all populations deviate from HWE subject to the same selection pressure). χ^2=10.6 (1 d.f.).

acid change, which has a rare allele frequency greater than 6%. For the rarer coding variants we did not have the statistical power to detect frequency differences between cases and controls. The odds ratios (OR) for breast cancer in each of the five case-control series in which we have analysed the N372H polymorphism are shown (Table 1). A joint analysis of all five series shows that the HH homozygotes, but not the heterozygotes, are associated with a significantly increased risk of breast cancer (OR [HH:NN]=1.31; 95% CI, 1.07–1.61). Even when the hypothesis generating set (series 1) is excluded, the results are still significant (OR [HH:NN] =1.28; 95% CI, 1.04–1.58). The ORs for pre-and post-menopausal women are similar (data not shown). We estimate that the population attributable risk of breast cancer due to the N372H polymorphism is 2.13% (95% CI, 0.48–3.34). Although a heterogeneity test on all five series reveals no significant differences (P=0.9, 8 d.f.), there is an indication that the HH genotype confers a higher relative risk in the British population samples (OR=1.46; 95% CI, 1.13–1.89) than in the Finns or Germans. This may indicate an interaction of this genotype with environmental or other genetic factors that are more prevalent in the UK.

During the analysis of the case-control studies, we noted that the genotype distribution in the controls tended not to fit Hardy-Weinberg equilibrium (HWE), and so this was further investigated. The comparison of fit to HWE for each of the control sets is shown (Table 2). In the adult females, aged from 24 to 75 years, there is a significant deviation from HWE (P=0.047, 5 d.f.), and strong evidence for a consistent selective pressure in all five series (P=0.0069, 1 d.f.). There are fewer of both homozygote groups than expected and more heterozygotes. Relative to the fittest heterozygote group, both homozygotes show a reduced fitness, the HH have an estimated fitness of 0.82. We investigated whether the apparent selection against homozygotes is occurring before or after birth by analysing a set of 2,345 DNA samples from newborn individuals. We allocated samples from males and females at random for analysis and genotyped them blind to sex. There is a significant difference between genotype distributions of the newborn males and females (P=0.0015). Females show an excess of heterozygotes and a deficit of homozygotes (P=0.06; Table 2) consistent with that seen in all five series of adult controls and thus indicating that this effect is already apparent at birth. By contrast, there is a deficit of male heterozygotes and an excess of homozygotes (P=0.001; Table 2). Relative to the heterozygote males, at birth, the NN group has a fitness of 1.15 and the HH group, 1.38. The apparently stable existence of the H allele, at a frequency of 0.27 in both sexes in the population, might be explained by opposing selection pressures on males and females. It is interesting to note that in a sample of chromosomally normal, human spontaneous abortions, a greater proportion of HH females were aborted (10.9%) compared with live born (6.5% newborn females; Table 2), with a different ratio in males (8.1% aborted versus 9.7% live born). Although too small to be conclusive, if confirmed, this could indicate that selection might be acting on the fetus rather than the gamete. Moreover, in transgenic mice homozygous for a truncated Brca2 protein, a greater proportion of those that survive to birth are male[4].

In the homozygous *Brca2*-truncated mice[4] and human *BRCA1* mutation carriers[5], there is an association of genotype with birth weight. In the newborn human population, however, we see no significant differences between the mean birth weights of the three genotype classes in either sex after correction for gestational age, although, as expected, the males have a significantly higher mean birth weight than the females (P<0.0001). Thus, we appear to be seeing a sex-specific differential selection associated with the N372H genotypes, acting *in utero*, which is not reflected in an effect on birth weight. These findings suggest a role for BRCA2 during human development, which is possibly modified in males and females by differences in the hormonal environment. Both *BRCA1* and *BRCA2* mRNA concentrations are increased in response to oestrogen stimulation, mediated through the oestrogen receptor, in breast cancer cells[6,7]. If the same applies in embryonic tissues, it is conceivable that *BRCA2* expression may be different in female embryos, which have higher endogenous oestrogen concentrations, than in males.

We do not believe these results are due to a genotyping artefact for two reasons. First, we obtained significant deviations from HWE in both males and females, in opposite directions, in a large study carried out blind with respect to sex. Second, we see 98% agreement of results in a subset of 800 samples genotyped by both TaqMan and allele-specific oligo hybridization. Whether the N372H change is directly responsible for the effects we observe is not yet certain: this change substitutes a basic residue for a neutral one and is thus non-conservative. Residues 290–453 of BRCA2 have been shown to interact with the histone acetyltransferase P/CAF before the transcriptional activation of other genes[8], but it is not yet known whether the N372H substitution affects this binding. Thus, although N372H may well be the causative variant, we cannot rule out linkage disequilibrium with another, as yet undiscovered variant in *BRCA2*, or indeed in another gene in the region. There are

eight other genes in a 1.4-Mb sequenced region around *BRCA2* (http://www.sanger.ac.uk/Software/Acedb) and one of these, *klotho* (*kl*), has a role in premature ageing[9,10]. Further studies to investigate the exact role of this residue in the structure and function of BRCA2 protein are required to understand the mechanism of these effects on both prenatal viability and breast cancer risk.

Methods

Patients and controls. We assessed associations between *BRCA2* and breast cancer risk in five population-based case-control series in populations of female people of European descent as follows.

We used a study of 234 consecutive breast cancer cases attending Addenbrooke's Hospital, Cambridge, for treatment between 1992 and 1995 and diagnosed below age 71 y. We compared these with a group of 266 randomly selected, anonymous, female controls between 47 and 76 y taken from the EPIC study[11], a population-based cohort study of diet and health.

We used a study of 1,667 patients ascertained through the East Anglian Cancer Registry, as part of the Anglian Breast Cancer Study, comprising all patients diagnosed below age 55 y since 1991 and still alive in 1996, together with all those under 65 y diagnosed between 1996 and 1999. We compared these with a second group of 1,201 female controls also selected at random from the EPIC cohort[11].

We also used a study of 450 cases and 228 controls derived from two UK population-based case-control studies of breast cancer. The first study was based on women diagnosed with breast cancer below the age of 36 y in the UK between 1982 and 1985. Cases in the second study were females diagnosed between 36 and 45 y in the Yorkshire, South Thames and Oxford health regions between 1988 and 1989. An equal number of unaffected controls, matched for age, sex and family doctor, were also collected at the same time. No direct controls were collected for the patients under 36 y.

We used a population-based case-control study, conducted in two geographical areas in southern Germany[12], consisting of 659 unselected breast cancer patients aged under 51 y and 866 age-, sex- and residence-matched controls from the same population.

Finally, we used a population-based case-control study from Kuopio, Finland, consisting of 449 breast cancer patients under 92 y collected by the Kuopio Breast Cancer Project between April 1990 and January 1996. Healthy population controls (453) were randomly selected from the Population Registry and matched to the breast cancer cases by age, sex and long-term place of residence.

Newborn DNA. DNA samples from unselected newborn babies have been collected, with maternal consent, since 1996 from the West Cumberland Hospital, Whitehaven as part of the North Cumbrian Community Genetics Project (NCCGP), which has been set up for genetic epidemiological research[13]. We obtained DNA with the relevant information on sex, birth weight and gestational age from 1,195 males and 1,150 females. All the sample data were coded to ensure anonymity.

Spontaneous abortion DNA. DNA from spontaneous abortions has been collected since 1998 at the Children's and Women's Health Centre of British Columbia as part of a study on the effects of confined placental mosaicism in spontaneous abortion. Comparative genomic hybridization[14] (CGH) was performed and routine cytogenetics was attempted in all cases. Ploidy was determined by flow cytometry in samples with normal CGH results. Here we have used DNA from all the diploid fetuses obtained, totalling 46 females and 37 males. All of the samples were coded to ensure anonymity.

Genotyping. We genotyped all samples for the BRCA2 N372H polymorphism using the ABI PRISM 7700 sequence detection system or "TaqMan"(Applied Biosystems). We carried out PCR on DNA (20 ng) using 1× TaqMan universal PCR master mix, forward (5′–CTGAAGTGGAACCAAATGATACTGA–3′) and reverse (5′–AGACGGTACAACTTCCTTGGAGAT3′) primers (300 nM), 100 nM VIC labelled probe (detecting H) (5′–TCAAATGTAGCACATCA GAAGCCCTTTGA3′) and 50 nM FAM labelled probe (detecting N) (5′–ATTCAAATGTAGCAAATCAGAAGCC CTTTGA3′) in a 25-μl reaction. The polymorphic base is underlined. Amplification conditions on an MJ Tetrad thermal cycler (GRI) were as follows: 1 cycle of 50 °C for 2 min, followed by 1 cycle of 95 °C for 10 min and finally 30 cycles of 95 °C for 15 s and 62 °C for 1 min. We read the completed PCRs on an ABI PRISM 7700 Sequence Detector and analysed them using the Allelic Discrimination Sequence Detection Software (Applied Biosystems). For the software to recognize the genotypes, we included eight of each non-template controls, H template and N template in each 96-well plate. We generated the H and N templates by cloning a heterozygote PCR using the TA cloning kit according to manufacturers instructions (Invitrogen). We designed TaqMan primers and probes using the Primer Express Oligo Design Software v1.0 (Applied Biosystems).

Statistical methods. We tested for HWE using the χ^2 test or the likelihood ratio test. As five different series were studied, we analysed these jointly to allow for different genotype frequencies in each unit. We calculated the OR using logistic regression. In addition, we calculated floating CIs for the ORs in Table 1 according to ref. 15. We tested for heterogeneity by comparing the likelihood when all the ORs in each series were assumed the same with the likelihood when they were independently estimated. We calculated the attributable risk according to reference 16. We calculated the CIs for the attributable risk using a bootstrap method. We assessed the effect of genotype on mean birth weight by ANOVA, after linear regression to correct for length of gestation.

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